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(56) Documents cited
GB 2163555 A **GB 1602134 A** **GB 1456599 A**
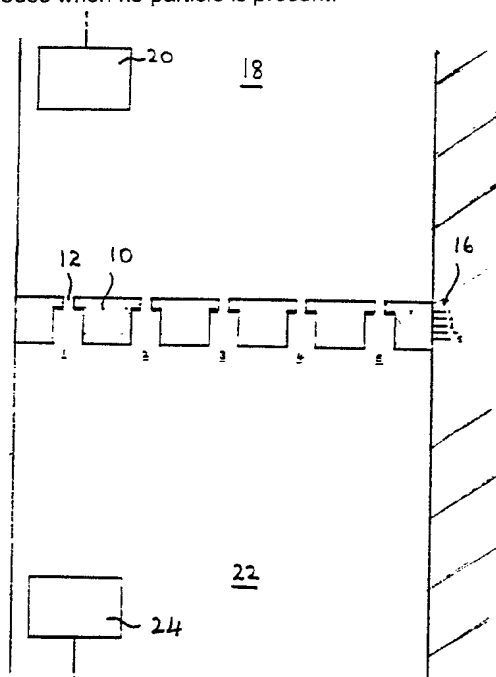**US 4438390 A** **US 4055799 A**

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(54) **Counting, volume and deformability measurement of particles and biological cells**

(57) An apparatus for counting particles and for determining their volume and deformability comprises:

- a membrane (10) having a first reservoir (18) on one side thereof containing a first electrode (20) and a second reservoir (22) on the other side thereof containing a second electrode (24);
- the membrane (10) having a plurality of pores (12) therethrough, which provide in use the only means for establishing electrical continuity between the first and second electrodes via electrically conductive liquid contained in the reservoirs, each pore comprising a first portion leading into a second portion; and
- a respective third electrode located at the junction of the first and second portions of each pore. The changes in electrical potential sensed at the respective third electrodes are indicative of the presence of a particle within the pore, the size of the particle, and its transit time within each portion of the pore. The portions may be of equal length and diameter or different. The resistance of any portion is sufficient to provide a measurable voltage drop between the third electrode and both the first and second electrodes when no particle is present.

Fig 2

The claims were filed later than the filing date within the period prescribed by Rule 25(1) of the Patents Rules 1982.
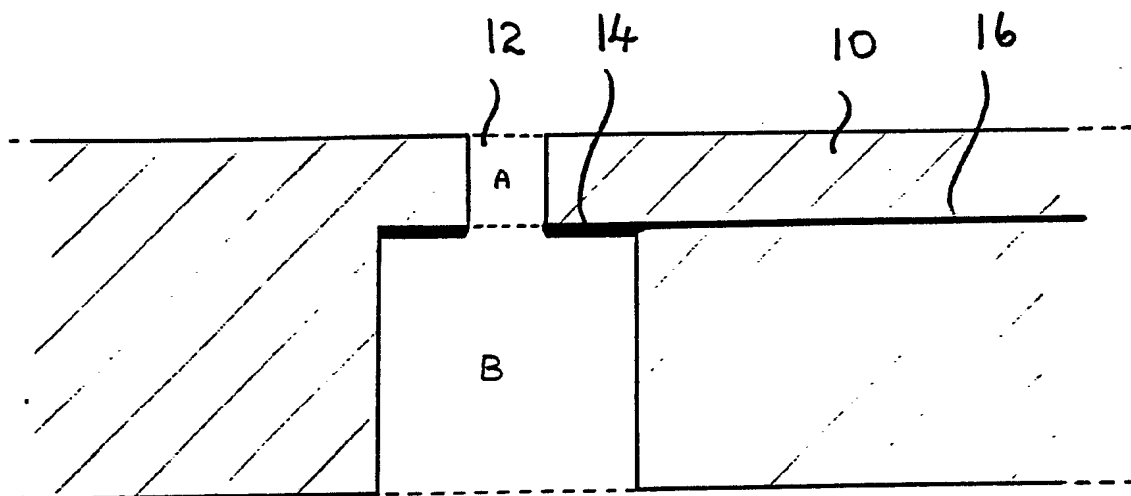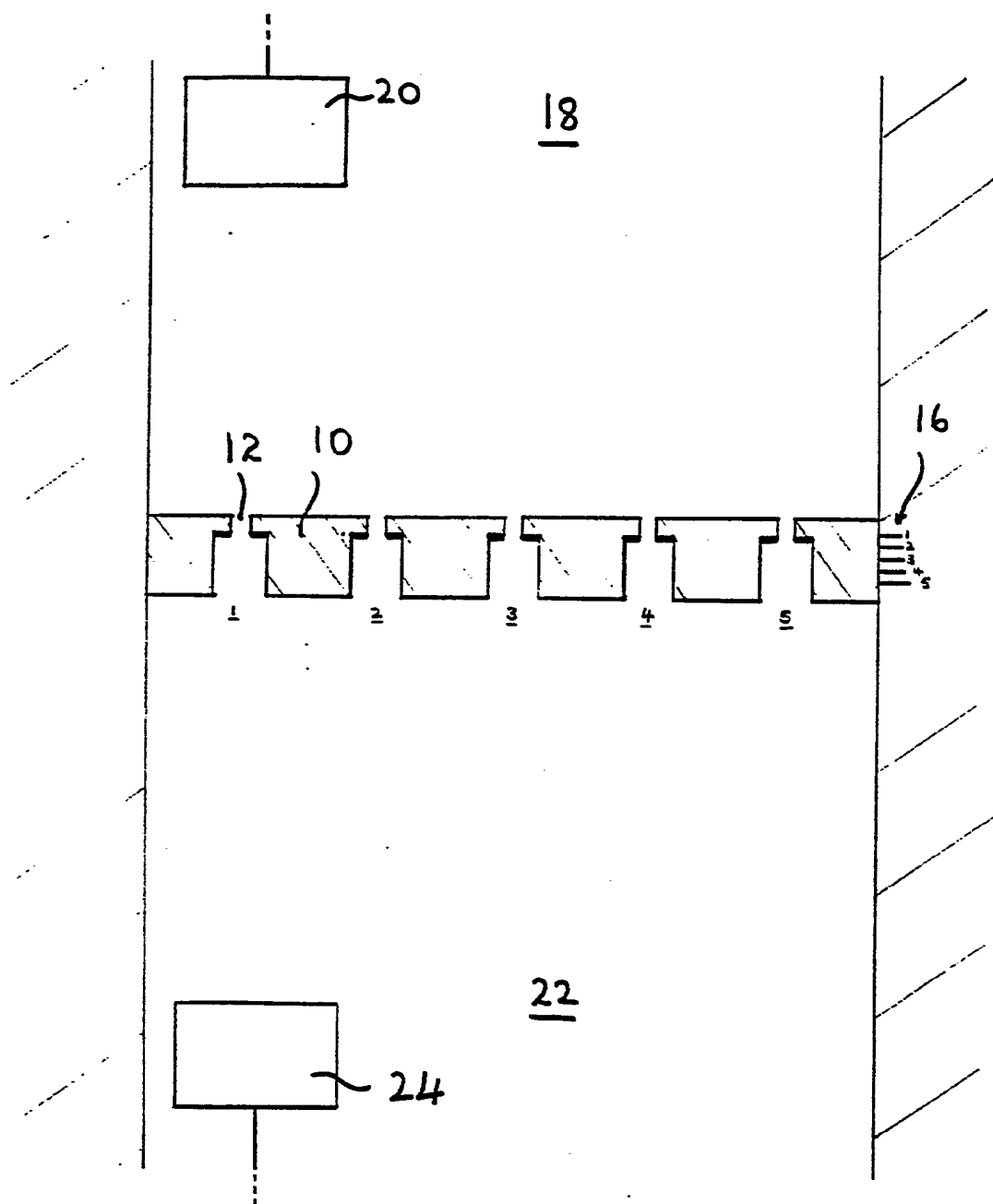
Fig 1

Fig 2

Fig 3

V_{IN} o———————

R_A

——o V_{OUT}

R_B

$0V$ o———————

Fig 4

V_{OUT}

ΔV

t

T

Fig 5

| PORE 1 | PORE 2 | PORE 3 | PORE 4 | PORE 5 |

V_{IN} o

R_{A1} R_{A2} R_{A3} R_{A4} R_{A5}
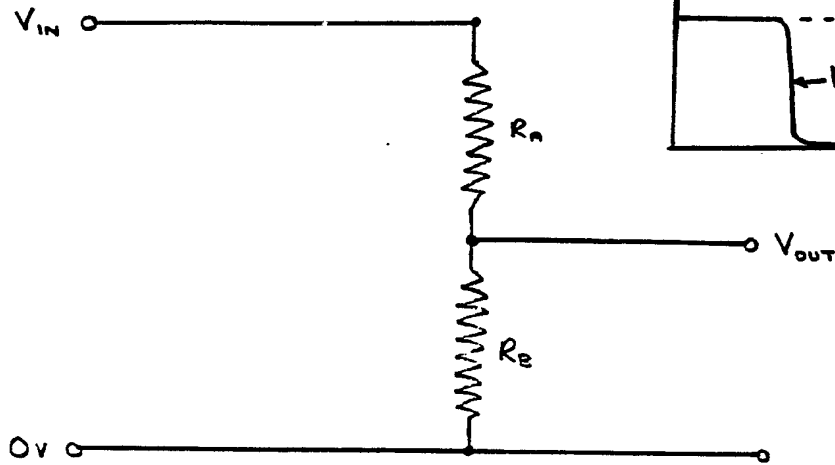
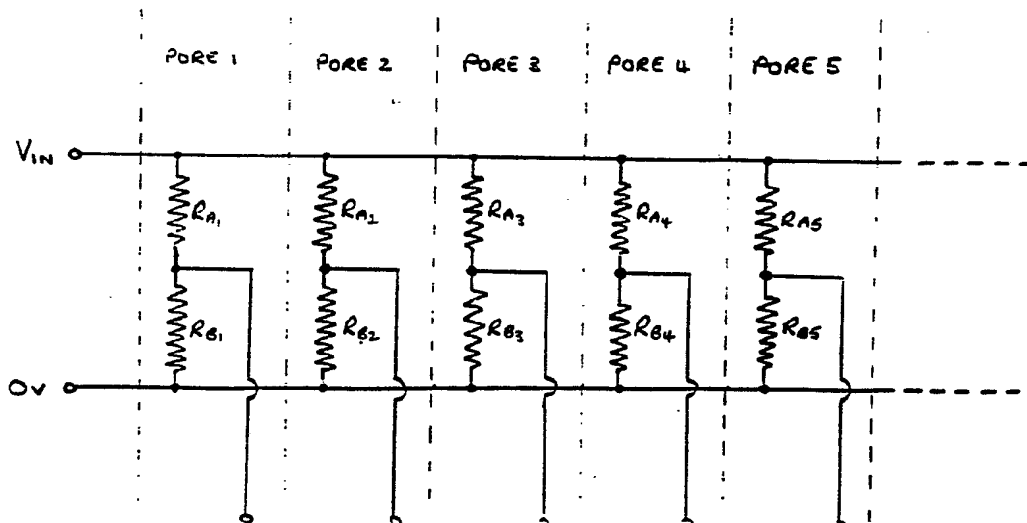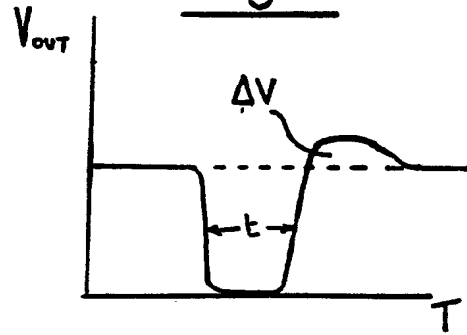R_{B1} R_{B2} R_{B3} R_{B4} R_{B5}

$0V$ o

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COUNTING AND VOLUME MEASUREMENT OF PARTICLES AND BIOLOGICAL CELLS, AND DETERMINATION OF THEIR DEFORMABILITY.

The present invention relates to an apparatus for counting, measuring the size, and determining the deformability of particles and biological cells suspended in a fluid. In particular, the apparatus allows for accurate counting and size measurement of particles in the size range from 10 nanometres upwards, using an adaptation of the known aperture impedance principle, and makes possible a high degree of noise elimination and reducing the problem of aperture blockage. The apparatus also provides an advantageous means to measure the deformability of inert particles and blood cells (both red and white blood cells) by measuring the time taken to pass through individual micropores.

The aperture impedance technique for counting and sizing particles suspended in electrolyte was first described in 1949 (W.H.Coulter US Patent 2656508). Since then the principle has been developed and refined and it is now the most commonly-used technique for the counting and sizing of particles of all descriptions. For accurate sizing, the diameter of the particle must lie between 2 and 60% of the aperture diameter. The smallest apertures used are typically 15 micrometres in diameter. The minimum size of particle that can be resolved using standard equipment is currently about 0.3 micrometres diameter. This limitation is mostly due to electrical

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noise and vibration (or acoustic noise) both of which
generate baseline fluctuations which obscure the signal
from smaller particles. Another important practical
difficulty, especially for small particles, is that the
small apertures are particularly prone to repeated
blockage by large particles, particle aggregates or
debris. Some specialised equipment has been constructed
which uses a single sub-micrometre pore to measure the
size and electophoretic mobility of particles down to 15
nanometres (DeBlois RW, Bean CP and Wesley RKA, J. Colloid
and Interface Science, 61 (2) pp. 323-335 1977), but this
also is affected by the foregoing disadvantages,
particularly pore blockage.

Measurement of particles in the nanometre size range
is essential in the control of industrial processes.
Variation in the size of particles of ceramics, metal
oxides, pigments, toners, powders, dyes, plastics,
photographic materials etc. will all influence
manufacturing processes and the characteristics of the
final product. The stability of colloids depends
critically upon particle size. Particles in the size
range 5nm to 1 micron are most commonly measured nowadays
by light scattering techniques, particularly photon
correlation spectroscopy, which are error prone, give
little information about particle size distribution, and
require immense computing power. Such instrumentation is
complex and expensive. It would be a particular advantage

to be able to extend the much simpler aperture impedance technique to the measurement of particles in this range.

Red blood cells are disc-shaped cells with a diameter of 7-8 micrometres. Their surface area is approximately 1.5 times greater than the minimum required to enclose their internal volume. This characteristic optimises the rate of oxygen transfer through the cell membrane and also contributes to their particular flexibility, or deformability. The deformability of red blood cells contributes to the ease with which blood flows in all blood vessels, but is of vital importance for flow in the smallest blood vessels in the body, the capillaries, which may have dimensions of 5 micrometres or less (i.e. very much smaller than red blood cells). Thus, the deformable nature of the cells is essential to permit blood flow through capillary networks to deliver oxygen to the tissues they supply.

Abnormal red blood cell rigidity is described in many diseases. Of particular interest are sickle cell anaemia and diabetes. In the former condition changes in deformability are of abrupt onset, with the cells changing to a rigid "sickle" shaped state. Blood flow in capillary networks is obstructed because the cells will no longer deform, causing extreme pain and tissue damage which may be severe. By contrast, the deformability change in diabetes is much less marked, but is chronic. The major debilitating complications of diabetes; blindness and

kidney failure, which develop slowly over period of years are known to result from capillary damage. Reduced red blood cell deformability, with consequent reduction to delivery of oxygen and nutrients, is believed to have a central role.

White blood cells though much fewer in number, are larger and much more rigid than red blood cells and consequently pass with even more difficulty through the smallest capillaries. Again, excessive white blood cell rigidity has been described in diabetes and also stroke.

Studies of red blood cell and white blood cell deformability in these and other disorders not only help to pinpoint the pathological processes involved, but are invaluable in the evaluation of drug treatments.

Numerous methods exist for the measurement of blood cell deformability. The various principles can be conveniently divided into two groups, (a) those which measure the average properties of a large number of cells and (b) those which make individual measurements on the cells.

(a) The main technique in this category is the filtration of a cell suspension through filters containing a large number (10^5 or more) of pores of smaller diameter than the cells (Leblond PF and Coulombe L, Scand. J. clin. Lab. Invest. 41, Suppl.156, pp.35-40 1981). The deformability is assessed by measuring either the flow rate of the suspension or the pressure

required to force the cells through the filter. The technique is simple, but is incapable of detecting the presence of subpopulations of more or less rigid cells. Variation of pore size and pore coincidence (even with the best commercially available filters) reduces precision.

The other technique of note is the measurement of the diffraction of a spot of light passed through a sheared suspension of blood cells (see Bessis M, and Mohandas N, Blood cells, 1, 307-313,1975). The shearing forces deform the cells, which diffract the light differently according to their state of deformation. Again, subpopulations cannot be discriminated.

(b) The standard method for measuring individual cell flexibilities is to aspirate each individual cell into a micropipette having an aperture smaller than the cell (Evans E. Biophys J., 30, 265-284, 1980). Deformability is assessed by observing the amount of cell membrane aspirated at a given pressure, or by measuring the transit time through the pipette aperture. In an equivalent technique (Keisewetter H, et al, Scand. J. Clin. Lab. Invest. 41, Suppl. 156, pp.229-232, 1981), the transit time of cells through a single minute pore in a membrane is measured. These techniques are more precise because only one aperture is used, and subpopulations of rigid cells may be identified. However, the disadvantage is that cells are aspirated

individually so it takes a long time to obtain sufficient data for analysis. Also, the membranes are expensive to produce and the single pore is repeatedly blocked by debris or leucocytes. An improvement upon this method is to measure the transit times of cells through a membrane with a larger number of pores (30 to 50), these transit times being measured by detecting the change in overall electrical resistance of the membrane as the cells pass through (Koutsouris D, et al, Biorheology, 25; pp 763-772, 1988). The technique is also described in British Patent specification 2163555. The advantages are that each cell transit is detected individually and that data is acquired much faster than with the micropipetting technique. There are several disadvantages:

i)   Coincident or nearly-coincident passage of two or more cells
through different pores gives rise to a spurious value for transit time - therefore cell suspensions must be very dilute to ensure that this is a rare event, and data from such events, if identifiable, must be rejected.

ii)   Pore blockage (or cells with very long transit times) causes a shift in the baseline conductivity which will be superimposed on the transits of other cells.

iii) The pores vary in size. The microporous filters used are made by the track/etch technique i.e.

irradiation of polycarbonate film by energetic particles, causing weakening of the structure along tracks, followed by etching along these tracks to produce holes of the correct dimensions. The random nature of the process results in a certain degree of pore coincidence, and the diameters of the pores have quite a wide degree of variation in diameter. Consequently, the transit times even of identical cells will vary if passing through different pores.

It is an object of the present invention to mitigate these disadvantages.

The present invention provides an apparatus for counting particles and for determining their volume and deformability which comprises:

- a membrane having a first reservoir on one side thereof containing a first electrode and a second reservoir on the other side thereof containing a second electrode;

- the membrane having a plurality of pores there through, which provide in use the only means for establishing electrical continuity between the first and second electrodes via electrically conductive liquid contained in the reservoirs, each pore comprising a first portion leading into a second portion; and

- a respective third electrode being located at the junction of the first and second portions of each

pore, in use the changes in electrical potential sensed at the respective third electrode being indicative of the presence of a particle within the pore, the size of the particle, and its transit time within each portion of the pore. When the particle size exceeds the pore size, the transit time provides an indication of the deformability of the particle.

As used herein, the term "membrane" means a thin sheet of rigid or flexible electrically insulating material or a composite sandwich of two or more such materials. The term "particle" is to be interpreted to include not only rigid particles but also deformable particles, such as cells.

The purpose of the first and second portions of the pore, with the third electrode positioned at the junction of these portions, is to create an electrical resistance between the first and third electrodes, and between the third and second electrodes. Generally, the particles and cells have a much lower electrical conductivity than the electrically conductive liquid (for example electrolyte solution) in which they are suspended. When the particle enters the first pore portion, the electrical resistance between the first and third electrodes increases thereby varying the voltage at the third electrode; a constant voltage being applied between the first and second electrodes. Continued passage of the particle into the second pore portion increases the electrical resistance between the third and second electrodes so varying again the voltage sensed at the third electrode, but in the

opposite sense. It is of particular significance that the
changes in resistance occurring in any one pore or region
thereof can have no influence on any other pore in the
membrane, thus all pores are both electrically and
physically independent of any other. Generally, the
electrical resistances in the two pore portions in the
absence of a particle will be arranged to be approximately
equal, but this is not essential - the only absolute
requirement is that the resistance of any portion is
sufficient to provide a measurable voltage drop between
the third electrode and both the first and second
electrodes when no particle is present. This may be
achieved by making the pore portions of equal length and
diameter. Alternatively, the first pore could be narrower
and shorter, whilst the second pore portion is wider and
longer. This latter arrangement is particularly useful
for the simultaneous measurement of blood cell size and
deformability, when the transit time of the cell through
the narrow first pore portion gives an indication of the
cell deformability, whilst the change in electrical
resistance in the second pore portion is proportional to
the volume of the cell.

The membrane is generally formed from a sandwich of
two electrically insulating materials between which are
sandwiched the third electrodes and their associated
leads. The third electrodes and the pores may be
fabricated by electron beam lithography. One means to

form the membrane is to prepare the third electrodes and leads on the surface of a thin glass sheet by electron beam lithography, to lay over these a coating of an etchable polymer, for example polyamide, through which the first portion of the pores is made (again by electron beam lithography) and finally to etch connecting channels through the glass to form the second portion.

For most applications, the number of pores in the membrane is in the region 10-100. Because the pores are physically and electrically independent, the maximum number of pores is limited only by the practical considerations of making electrical connections to the third electrodes and analysing the resulting signals. Thus, a membrane with thousands of pores is feasible. In fact, it is not necessary to actually use all of the pores present, any number may be left unconnected. Similarly, pores can be ignored or disconnected if they become blocked.

Generally, the same electrically conductive liquid will be placed in both reservoirs. For the analysis of biological cells, this solution will be such as to maintain the cells intact and substantially unchanged.

The invention also relates to a corresponding method for counting particles and for determining their volume and deformability in which:

- a suspension of the particles or cells in a suitable conducting electrolyte solution is placed in one or other of the reservoirs, with identical particle-free

solution placed in the other;

- a pressure difference is applied across the membrane to induce the flow of the liquid containing the suspended particles or cells into and through the pores (this pressure depends upon the application, but is typically in the range of 0.1 to 30 millibar);

- the electrical potentials sensed at the respective third electrodes are fed into a signal processing device programmed to extract relevant parameters according to the particular application (such as transit times and voltage changes) and to convert these into the number of particles and their volumes, or the size and deformability of cells.

An embodiment of the present invention will now be described by way of example only with reference to the accompanying drawings wherein;

Figure 1 is an enlarged schematic cross-sectional view of a single pore;

Figure 2 is a schematic cross-sectional view of the apparatus including five membrane pores;

Figure 3 is a representation of the electrically equivalent circuit to each pore;

Figure 4 indicates a typical voltage trace between the third and second electrodes as a blood cell passes through the pore; and

Figure 5 indicates an electrically equivalent circuit for the five pore apparatus of Figure 2.

The membrane shown in Figure 1 comprises a thin sheet of rigid or semi-flexible electrically insulating membrane 10. The membrane may be formed of a single sheet or a composite sandwich of two or more sheets of the same or different materials. A pore 12 passes through the membrane and comprises a first portion A and a second wider and longer portion B. At the junction of portions A and B is arranged a third electrode 14 and conductive lead 16. The dimensions of each of the two portions of the pore are chosen with regard to the ratio of length to a cross-sectional area, such that the resistances R_a and R_b are of the same order of magnitude.

Figure 2 shows the whole apparatus comprising the membrane 10 and five pores 1 to 5 therethrough, each having its respective output lead 16. The membrane separates a first electrolyte reservoir 18 containing a first electrode 20 from a second electrolyte reservoir 22 containing a second electrode 24. The only electrical contact between the reservoirs is via the membrane pores.

Figure 3 shows the electrically equivalent circuit to each single pore. In use, a voltage V_{in} is applied across the first and second electrodes 20,24. In the absence of a particle in the pore, the resistance R_a of the first pore portion is substantially the same as resistance R_b of the second pore portion, such that V_{out} is approximately half V_{in}. The electrically equivalent circuit for the five pore membrane shown in Figure 2 is given in Figure 5.

The fundamental advantage of this arrangement is that events occurring at each pore can be recorded individually, and that each pore is electrically independent of any other.

Operation

In use, an appropriate voltage, for example 5 volts, is applied between the electrodes in each reservoir as described above. A suspension in electrolyte of the particles to be analysed is introduced into one of the reservoirs, 18, and is aspirated under a pressure between -0.1 to -30 millibar, for example -5 millibar, into the other reservoir 22. Particles pass through with the electrolyte, and (since they have a lower conductivity than the electrolyte) cause an increase in the resistance of the pore which varies the voltage detected by the third electrode. The precise form of the voltage change depends upon the application:

- if biological cells are to be analysed for their deformability and volume, then the system will be arranged such that the diameter of the first part of the pore is significantly smaller than that of the cell, for example 3 to 5 micrometres for red blood cells, so that the cell must deform in order to pass through. A typical trace for the change in voltage detected between the third and second electrodes during the passage of a blood cell is illustrated in Figure 4. The voltage V is steady at its resting

level until the cell enters portion A. The cell takes a finite time to squeeze through the narrow pore portion, and effectively occludes it, causing its resistance R_A to rise substantially, reducing the voltage V to near zero. As the cell emerges from portion A into portion B, which has a larger diameter than the cell, it causes a smaller increase in resistance R_B so that voltage V rises above the steady state level. The difference in voltage above the steady-state level is a measure of the volume of the cell.

- if the number and size of small particles is to be determined, the system will be arranged so that the pore size used is such that the expected particle size range of interest lies within 2-60% of the pore diameter. In this case, the change in resistance R_A is not so large as if the pore was completely occluded, and the difference in voltage below the steady-state level is a measure of the volume of the particle. Similarly, when the particle enters region B, the difference in voltage above the steady-state level is again a measure of the particle volume. Also, since each particle passing through a pore causes a change in voltage, the number of particles can easily be counted. For this application, the particles might also be propelled through the pores by electrophoretic or electro-osmotic potentials instead, or in addition to, the trans-membrane pressure difference.

ADVANTAGES:

The specific advantages of this device arise from the independence of each pore in the membrane, which may be considered to comprise a large number of detectors operating in parallel. They are:

1) In respect of its use for particle counting and size analysis:

(i) NOISE REDUCTION:

The present device mitigates the problem of electrical noise and vibration by the use of multiple independent pores as apertures. A number (probably 30-50, possibly many more) of these pores, of suitable diameter, are preferably arranged to be physically close together in the membrane. In this situation, any external influences, such as electrical noise and vibration, will affect each pore in a similar manner. A spurious signal arising as a consequence of noise will be detected in the output from the probe electrode of many or all of these pores. However, when a particle passes through a pore, it is only the output from that pore which is affected. This valid signal may therefore be extracted from the noise by comparing the output from an individual pore with that from its surrounding neighbours. There are various ways to achieve this; for example subtraction from the occupied pore's signal of an averaged background signal derived from the unoccupied pores, or by simply

disregarding the signal from the occupied pore if a
simultaneous spurious signal is detected in the
unoccupied pores.

(ii) REDUCTION OF THE CONSEQUENCES OF APERTURE
BLOCKAGE: Aperture occlusion, either partial or
total, can cause much difficulty in the known aperture
impedance technique. With the present device, such
occlusion is easily detected by a permanent change in
the output level of the pore affected, and any
subsequent signal from that pore is then disregarded.
Because of the large number of independent pores there
is a high degree of redundancy, and many may be
allowed to become occluded before the operation must
be abandoned and the pores cleared.

(iii) A range of different pore sizes may be
incorporated into a single device in order to increase
the size range of particles measurable. For example,
the device may incorporate ten 50 micrometre pores
together with one hundred of 5 micrometre diameter.

2) In respect of its use for blood cell deformability
determination:

    i)   High cell concentrations may be used, because the
system is indifferent to the simultaneous presence of
cells in two or more pores, which leads in turn to a
high rate of data acquisition.

    ii) Permanent pore blockage, or cells with long
transit times, present no difficulty because of the

pore independence; a blocked pore will not affect the output from any of the others which will still operate normally.

iii) A series of pores having different sizes and cross-sectional shapes may be incorporated into a single membrane. For example, one might wish to use a set of pores of 2,4,6,8, and 10 micrometre diameter, perhaps ten of each size; or possibly relatively more of the smaller sizes to compensate for the longer transit times. Alternatively, different cross-sectional shapes could be used in order to obtain some information about the relative contributions of the cell membrane and cytoplasm to the deformation.

iv) The size of each cell can be measured as well as its transit time. The combination of these two parameters is particularly valuable, because it is well established that the passage of cells through pores depends both upon the overall flexibility and the cell volume.

v) Errors in the output due to external influences, such as electrical noise and vibration, may be eliminated as described in the previous section.

CLAIMS:

1.   An apparatus for counting particles and for determining
their volume and deformability which comprises:

-       a membrane having a first reservoir on one side thereof
        containing a first electrode and a second reservoir on
5   the other side thereof containing a second electrode;

-       the membrane having a plurality of pores therethrough,
        which provide in use the only means for establishing
        electrical continuity between the first and second electrodes
        via electrically conductive liquid contained in the reser-
10   voirs, each pore comprising a first portion leading into
        a second portion; and

-       a respective third electrode being located at the junction
        of the first and second portions of each pore, in use
        the changes in electrical potential sensed at the respective
15   third electrode being indicative of the presence of a par-
        ticle within the pore, the size of the particle, and its
        transit time within each portion of the pore.

2.   An apparatus according to Claim 1 in which the resistence
of any pore portion is sufficient to provide a measureable voltage
20   drop between the third electrode and both the first and second
electrodes when no particle is present.

3.   An apparatus according to Claim 2 in which the pore portions
are of equal length and diameter.

4.   An apparatus according to Claim 2 in which the first
25   pore portion is narrower and shorter, whilst the second pore
portion is wider and longer.

5. An apparatus according to any of the preceding Claims in which the membrane is formed from a sandwich of two electrically insulating materials between which are sandwiched the third electrodes and their associated leads.

5   6. An apparatus according to Claim 5 in which the third electrodes and the pores may be fabricated by electron beam lithography.

7. A method for counting particles and for determining their volume and deformability in which:

10   -   a suspension of the particles or cells in a suitable conducting electrolyte solution is placed in one or other of the reservoirs, with identical particle-free solution placed in the other;

     -    a pressure difference is applied across the membrane
15   to induce the flow of the liquid containing the suspended particles or cells into and through the pores (this pressure depends upon the application, but it typically in the range of 0.1 to 30 millibar); and

     -    the electrical potentials sensed at the respective third
20   electrodes are fed into a signal processing device programmed to extract relevant parameters according to the particular application (such as transit times and voltage changes) and to convert these into the number of particles and their volumes, or the size and deformability of cells.

25   8. An apparatus for counting particles and for determining their volume and deformability substantially as described herein with reference to and as illustrated in the accompanying drawings.

9. A method for counting particles and for determining their

volume and deformability, substantially as described herein with reference to and as illustrated in the accompanying drawings.